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AUTHOR(S):

Yamashita, Kouhei; Miyoshi, Takashi; Arai, Yasuyuki; Mizugishi, Kiyomi; Takaori-Kondo, Akifumi; Ueyama, Takehiko

Enhanced generation of reactive oxygen species by interferon-γ may have contributed to successful treatment of invasive pulmonary aspergillosis in a patient with chronic granulomatous disease

Kouhei Yamashita[1,*], Takashi Miyoshi[1], Yasuyuki Arai[1], Kiyomi Mizugishi[1], Akifumi Takaori-Kondo[1], Takehiko Ueyama[2]

[1]Department of Hematology and Oncology, Graduate School of Medicine, Kyoto University, Kyoto 606-8507, Japan, and [2]Laboratory of Molecular Pharmacology, Biosignal Research Center, Kobe University, Kobe 657-8501, Japan.

[*]Corresponding author: Kouhei Yamashita, M.D., Ph.D. Department of Hematology and Oncology, Graduate School of Medicine, Kyoto University, 54 Shogoin Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan. Tel.: +81-75-751-4290; Fax: +81-75-751-4963; E-mail: kouhei@kuhp.kyoto-u.ac.jp

Running head: Augmented ROS production by IFN-γ in CGD

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Abstract

Invasive pulmonary aspergillosis (IPA) is a life-threatening complication of chronic granulomatous disease (CGD), a rare inherited disorder of phagocytes that is characterized by a defect in the production of reactive oxygen species (ROS) caused by mutations in NADPH oxidase 2. Here, we report a case of successful treatment of IPA complicated with CGD by the administration of interferon-γ (IFN-γ) in combination with voriconazole. The patient carried a splice site mutation in the CYBB gene, and the neutrophils could produce a certain amount of ROS. In this case, augmentation of ROS generation in the patient's neutrophils was observed after in vivo IFN-γ treatment, which may be attributable to the induction of a normal CYBB gene in the myeloid progenitor cells. This treatment, in combination with voriconazole, may have contributed to the reversal of IPA in this patient. These results suggest that the in vivo use of IFN-γ may augment ROS generation in CGD neutrophils, thus leading to the successful treatment of severe IPA.

Keywords: chronic granulomatous disease, invasive pulmonary aspergillosis, interferon-γ, reactive oxygen species, voriconazole

Introduction

Chronic granulomatous disease (CGD) is a rare inherited disorder caused by mutations in NADPH oxidase 2 (Nox2) of phagocytes. CGD neutrophils show a defect in the production of reactive oxygen species (ROS), leading to recurrent bacterial and fungal infections. Mutations in 5 genes (*CYBB, CYBA, NCF1, NCF2, NCF4*) have been reported to cause CGD [1]. Mutations of *CYBB* in Xp21.1, which codes for gp91^{phox}, also known as Nox2, account for almost 80% of CGD in Japan. Mutations in the *CYBB* gene, which encompasses 13 exons, are heterogeneous, including missense and nonsense mutations, deletions, insertions, and splice site mutations [2]. The use of various cytokines has been attempted to enhance the ability of ROS generation of CGD neutrophils. A previous report demonstrated that interferon (IFN)-γ improved the superoxide-generating ability by changing the splicing pattern of transcripts in CGD neutrophils with a splice site mutation (G to A point mutation at nucleotide 252 in exon 3) in the *CYBB* gene [3].

Invasive pulmonary aspergillosis (IPA) is one of the life-threatening complications of CGD, since ROS play a critical role in killing *Aspergillus* hyphae. Voriconazole, which is a triazole antifungal medication, has become the standard treatment for IPA. However, it leads to an insufficient response in some CGD cases, for instance, because of the emergence of multi-azole-resistant *Aspergillus* [4]. On the other hand, a previous observation showed that treatment with human recombinant IFN-γ augmented the ability of CGD neutrophils to generate ROS, resulting in decreased susceptibility to bacterial and fungal infections [5]. In addition, it was reported that *in vivo* IFN-γ therapy augmented the *in vitro* ability of CGD neutrophils to kill *Aspergillus* hyphae [6]. Therefore, IFN-γ treatment is expected to exert an additive therapeutic effect on the CGD patients in critical conditions.

We recently encountered a CGD patient with a splicing site mutation in the *CYBB* gene, who was complicated by severe IPA. In this case, IFN-γ treatment in combination with voriconazole markedly improved the symptoms and clinical data. We observed the enhancement of ROS production of the CGD neutrophils after IFN-γ treatment as revealed by flow cytometric analysis, possibly contributing to the successful killing of *Aspergillus*.

Case report

A 26-year-old Japanese male was admitted to our hospital because of fever, cough, and dyspnea on August 29, 2007 (day 0). He had been exposed to a large amount of dirty dust three days before the admission. He had been diagnosed with gp91^{phox}-deficient CGD in his early childhood by gene mutational analysis. It revealed a G to A point mutation at nucleotide 252 in exon 3 of the *CYBB* gene, which produces an aberrant splice donor site in exon 3 (Ggt to Agt), as previously described [3]. He had suffered from recurrent bacterial infections, such as meningitis and pneumonia from his early childhood. His maternal grandfather and cousin were also CGD patients. On admission, his temperature was 37.3°C and percutaneous oxygen saturation was 78% under room air. Coarse crackles were heard in the bilateral lung fields. Laboratory data are shown in Table 1. Neutrophilic leukocytosis and elevation of the CRP value were observed. On admission, chest X-ray film showed bilateral infiltrations (Fig. 1a), and a chest CT scan demonstrated diffuse small granular opacities and consolidations in a lobular-centric manner in the bilateral lung fields, suggesting military tuberculosis (Fig. 1b). Bronchial alveolar lavage (BAL) showed *Aspergillus fumigatous* (1+), and the quantitative analysis of *Aspergillus* was 3 x 10^5 copies/ml. The polymerase chain reactions (PCR) for *Mycobacterium (M) tuberculosis*, *M. avium complex*, and *M. intracellulare* DNAs were all negative in BAL, bone marrow, and gastric fluid specimens. The serum β-D glucan level was 10.98 pg/ml. Other microbial pathogens including *pneumococcal pneumonia*, *Chlamydia pneumonia*, *Legionella pneumophilia*, *Mycoplasma*, and *Cytomegalovirus*, were not detected. Based on these findings, a diagnosis of invasive pulmonary aspergillosis was made. The clinical course is shown in Fig. 2. The treatment with sulfamethoxazole (SMX)/trimethoprim (TMP) and voriconazole started on day 0. Short-term additional antibiotic treatments with vancomycin, cefepime, and clarithromycin were performed. Moreover, treatment with a high concentration of oxygen (7 L/min) was needed on admission. However, these treatments did not have enough effect on his clinical course, as revealed by the laboratory data with persistent leukocytosis and elevated CRP level (Fig. 2). Thus, after day 7, subcutaneous interferon (IFN)-γ (2.5 x 10^5 units/m^2) was administered three times a week for the first week and once a week for the next 6 weeks. After the start of IFN-γ therapy, the white blood count and CRP value dropped (Fig. 2), and the consolidations in the bilateral lung fields on the CT scan

markedly improved on day 13 (Fig. 1c). The oxygen was tapered off over 10 days. The patient was discharged on day 13. The pulmonary consolidations on the CT scan were further improved on day 36 (Fig. 1d).

Materials and Methods

Isolation of neutrophils

Human neutrophils were isolated from the peripheral blood of a healthy adult volunteer and the CGD patient by sedimentation through a 2-step Percoll gradient, as previously described [7]. The healthy volunteer and CGD patient provided written informed consent for participation in an institutional review board-approved protocol at Kyoto University Hospital.

ROS generation of neutrophils before and after the IFN- γ treatment by flow cytometry

Neutrophils (2 x 10^5 cells) were loaded with 2 μM dihydrorhodamine 123 (DHR, Molecular Probes) for 5 min at 37°C. After that, cells were stimulated with 100 ng/ml phorbol myristate acetate (PMA, Sigma-Aldrich) for 15 min at 37°C and analyzed by flow cytometry.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA from neutrophils was isolated using Trizol (Invitrogen). Total RNA (1 μg) was reverse-transcribed using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) following the manufacturer's instructions. PCR for a portion of *CYBB* alleles was performed using the following primers: P1 (5'- TGCCACCATGGGGAACTGGGCTGTGAATGAG -3') on exon 1 and P2 (5'- GTACCAAAAGACTTCAAAGTAAGACCTCCGGATG -3') on exon 6. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH)* was used to monitor RNA recovery. The PCR was performed under the following conditions: 94 ˚C for 2 min followed by 40 cycles of 94 ˚C for 30 sec, 63 ˚C for 45 sec and 72 ˚C for 1 min.

ROS generation by Nox2 mutants lacking exon 3

The wild-type Nox2, named wt Nox2(1-13), was constructed by inserting the cDNA fragment containing the entire open reading frame of human *Nox2*, which consists of 13 exons, in-frame into pcDNA3.1. A series of *Nox2* mutants lacking exon 3 were generated by inserting the relevant exon regions in-frame into pcDNA3.1. These deletions were as follows: Nox2(1,2,4-13)(exon 1, exon 2 and exons 4-13), Nox2(1,4-13)(exon 1 and exons 4-13), Nox2(1,4,6-13)(exon 1, exon 4 and exons 6-13), Nox2(1,6-13)(exon 1 and exons 6-13) and Nox2(1,7-13)(exon 1 and exons 7-13). ROS generation from HEK293 (ATCC) cells transfected with wild-type Nox2 or mutant Nox2 in combination with $p47^{phox}$ and $p67^{phox}$ was measured by a luminol-enhanced chemiluminescence method in the presence of horseradish peroxidase (HRP) and luminol, as previously described [8].

Results and Discussion

The intensity of DHR fluorescence of CGD neutrophils immediately before IFN-γ treatment (day 7) upon stimulation with PMA apparently diminished compared with that of healthy neutrophils (Fig. 3a, b, d). After IFN-γ treatment (day 50), CGD neutrophils showed a higher intensity of DHR fluorescence by PMA stimulation, which was similar to that of healthy neutrophils (Fig. 3b, c, d), suggesting that IFN-γ treatment augmented the ROS production of CGD neutrophils *in vivo*. This finding was compatible with a previous report, where the authors demonstrated the effect of IFN-γ *in vivo* on CGD patients under healthy conditions [3]. By contrast, in our case, the therapeutic effectiveness of IFN-γ was observed in a CGD patient complicated with IPA, where it possibly contributed to the clinical improvement by the enhancement of microbicidal activity.

In order to clarify the mechanism of how IFN-γ enhanced the activity of ROS production in our CGD neutrophils, we analyzed the ROS production from HEK293 cells transfected with plasmid containing various types of mutant Nox2, which lack exon 3. The mutation in our patient was a G to A silent mutation (Ala to Ala) at nucleotide 252 in exon 3. However, this mutation site is adjacent to the splice junction, making splicing-variant transcripts skipping exon 3 [3]. Nunoi et al. proposed the hypothesis that IFN-γ corrects a part of aberrant splicing of transcripts in the progenitor cells, which can produce ROS to some extent, and induces a prolonged effect on ROS generation activity. Thereby, a

considerable amount of the corrected progenitor cells produce mature neutrophils with functional gp91^{phox} in CGD patients with this mutation [3, 9]. In our experiment, none of the HEK293 cells transfected with mutant Nox2, which lack exon 3, showed any significant ROS generation in response to PMA stimulation as revealed by luminol-enhanced chemiluminescence method. Only HEK 293 cells transfected with wild-type Nox2 (wt Nox2(1-13)) could produce ROS upon stimulation with PMA, which was suppressed by the addition of diphenyleneiodonium (DPI), a Nox inhibitor (Fig. 4). Furthermore, *in vitro* administration of IFN-γ did not directly affect the ROS production of neutrophils from the CGD patient (data not shown). On the other hand, we performed RT-PCR analysis for *CYBB* gene transcripts from patient's neutrophils under favorable health conditions. The amplified transcripts using primers on exon1 and exon 6 exhibited a 518-bp band that lacked exon 3 (Fig. 5a, b, c)[3]. Unfortunately, we were not able to check the splicing-variant transcripts of the patient's neutrophils immediately before and after IFN-γ treatment, since the quality of RNAs obtained was not favorable. However, the mutation in our case is exactly the same as the one in the previous report by Ishibashi et al., it is rational to assume that the similar splicing events had happened after IFN-γ treatment [3]. Based on these findings, we speculate that IFN-γ, at least in part, precluded the exon 3 skipping in the progenitor cells. As a result, a considerable amount of neutrophils derived from the progenitor cells may have generated a full-length transcript of Nox2, irrespective of the presence of G to A substitution in exon 3, and acquired the activity of ROS production. This speculation is supported by a report that -1 position (G) of splice donor consensus sequence (G/gt is 73 % of all splice donor site) is A/gt, T/gt and C/gt in 9 %, 12 % and 6 %, inspite of 100% universality at +1 and +2 positions (gt) [10]. It remains unclear why the patient's neutrophils produced a cerain amount of ROS before treatment.

Many clinical observations in CGD patients provide evidence that ROS are important for killing *Aspergillus* hyphae [11, 12]. Neutrophils, which engulf *Aspergillus* hyphae in the phagosome, can kill them efficiently in a ROS-dependent manner. Recently, neutrophil extracellular traps (NETs), which capture bacteria, fungi, and viruses in extracellular structures consisting of DNA fibers and antimicrobial granule proteins, have been reported to play a pivotal role in innate host defense [13]. Previous reports show that CGD neutrophils are not able to make NETs, indicating that Nox activation of neutrophils is required for NET formation [14, 15]. The defect of NET formation in CGD neutrophils is likely to

contribute to failure to kill *Aspergillus*, although we did not examine NET formation in this patient.

It has been reported that the oral administration of itraconazole is effective for prophylaxis against aspergillosis in CGD patients [16]. Unfortunately, our patient was affected with IPA, since he did not take itraconazole. Voriconazole is now the first choice for the treatment of aspergillosis; however, reports of multi-azole-resistant *Aspergillus fumigatus* have been accumulated [4, 17]. A previous report demonstrated that another cytokine, granulocyte colony-stimulating-factor or granulocyte-macrophage colony-stimulating-factor, enhanced the candidacidal activity of neutrophils in collaboration with voriconazole [18], but it is unclear whether this is applicable to the killing activity of CGD neutrophils against *Aspergillus*. This study supports the idea that the *in vivo* enhancement of microbicidal activity in neutrophils by IFN-γ administration is effective in CGD patients with intractable fungal infections.

Conflict of interest

The authors declare no competing financial interests.

References

1. Segal BH, Leto TL, Gallin JI, Malech HL, Holland SM. Genetic, biomedical, and clinical features of chronic granulomatous disease. *Medicine (Baltimore)*. 2000;79:170-200.

2. Ishibashi F, Nunoi H, Endo F, Matsuda I, Kanegasaki S. Statistical and mutational analysis of chronic granulomatous disease in Japan with special reference to gp91-*phox* and p22-*phox*. *Hum Genet*. 2000;106:473-481.

3. Ishibashi F, Mizukami T, Kanegasaki S, Motoda L, Kakinuma R, Endo F et al. Improved superoxide-generating ability by interferon-γ □due to splicing pattern change of transcripts in neutrophils from patients with a splice site mutation in *CYBB* gene. *Blood*.2001; 98:436-41.

4. Hodiamont CJ, Dolman KM, Ten Berge IJ, Melchers WJ, Verweij PE, Pajkrt D. Multiple-azole-resistant Aspergillus fumigatus osteomyelitis in a patient with chronic granulomatous disease successfully treated with long-term oral posaconazole and surgery. *Med Mycol*. 2009;47:217-20.

5. Ezekowitz RA, Orkin SH, Newburger PE. Recombinant interferon gamma augments phagocyte superoxide production and X-chronic granulomatous disease gene expression in X-linked variant chronic granulomatous disease. *J Clin Invest*.1987;80:1009-16.

6. Rex JH, Bennett JF, Gallin JI, Malech HL, DeCarlo ES, Melnick DA. In vivo interferon-γ therapy augments the in vitro ability of chronic granulomatous disease neutrophils to damage *Aspergillus* hyphae. *J Infect Dis*.1991;163:849-52.

7. Oka S, Sasada M, Yamamoto K, Nohgawa M, Takahashi A, Yamashita K et al. Nitric oxide derived from human umbilical vein endothelial cells inhibits transendothelial migration of neutrophils. *Int J Hematol*.2005;81:220-7.

8. Ueyama T, Nakakita J, Nakamura T, Kobayashi T, Kobayashi T, Son J et al. Cooperation of $p40^{phox}$ with $p47^{phox}$ for Nox2-based NADPH oxidase activation during Fcγ receptor (FcγR)-mediated phagocytosis: mechanism for acquisition of $p40^{phox}$ phosphatidylinositol 3-phosphate (PI(3)P) binding. *J Biol Chem*. 2011;286;40693-705.

9. Nunoi H, Ishibashi F, Mizukami T, Hidaka F. Clinical evaluation of interferon-gamma treatment to chronic granulomatous disease patients with splice site mutations. *Jpn. J Infect Dis*. 2004;57:S25-6.

10. Mount SM. A catalogue of splice junction sequences. *Nucleic Acids Res.* 1982:10;459-72.

11. Mamishi S, Parvaneh, Salavati A, Abdollahzadeh S, Yeganeh M. Invasive aspergillosis in chronic granulomatous disease: report of 7 cases. *Eur J Pediatr*. 2007;166:83-4.

12. Gallin JI, Zarember K. Lessons about the pathogenesis and management of aspergillosis from studies in chronic granulomatous disease. *Trans Am Clin Climatol Assoc*. 2007;118:175-85.

13. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS et al. Neutrophil extracellular traps kill bacteria. *Science*. 2004;303:1532-5.

14. Fuchs TA, Abed U, Goosmann C, Hurwitz R, Schulze I, Wahn V et al. Novel cell death program leads to neutrophil extracellular traps. *J Cell Biol*. 2007;176:231-41.

15. Nishinaka Y, Arai T, Adachi S, Takaori-Kondo A, Yamashita K. Singlet oxygen is essential for neutrophil extracellular trap formation. *Biochem Biophys Res Commun*. 2011;413:75-9.

16. Gallin JI, Alling DW, Malech HL, Wesley R, Koziol D, Marciano B et al. Itraconazole to prevent fungal infections in chronic granulomatous disease. *N Engl J Med*. 2003;348:2416-22.

17. Howard SJ, Arendrup MC. Acquired antifungal drug resistance in Aspergillus fumigatus:

epidemiology and detection. *Med Mycol*. 2011;49 Suppl 1:S90-5.

18. Vora S, Purimetla N, Brummer E, Stevens DA. Activity of voriconazole, a new triazole, combined with neutrophils or monocytes against Candida albicans: effect of granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor. *Antimicrob Agents Chemother*. 1998;42:907-10.

Figure legends

Fig. 1

a, b Chest X-ray film (**a**) and CT scan (**b**) on admission (day 0). Diffuse small granular opacities and consolidations in the bilateral lungs were observed. **c, d** Chest CT scans on day 13 (**c**) and day 36 (**d**). Note that the consolidations in the bilateral lung fields on the CT scan markedly improved after IFN-γ treatment.

Fig. 2

Clinical course of the patient. Abbreviations; ST: sulfamethoxazole (SMX)/trimethoprim (TMP), VRCZ: voliconazole, CFPM: cefepime, VCM: vancomycin, CAM: clarithromycin.

Fig. 3

Analysis of ROS production of neutrophils by the DHR assay. **a, b** ROS production of neutrophils from the patient immediately before IFN-γ treatment (day 7) (**a**) and healthy control at the same time (**b**). **c** ROS production of neutrophils from the patient after IFN-γ treatment (day 50). The histograms show ROS production by neutrophils upon stimulation with PMA. The logarithmic fluorescence intensity is shown on the *x*-axis and the cell count on the *y*-axis. Filled and open histograms indicate PMA-stimulated and unstimulated neutrophils, respectively. **d** Quantitative analysis of ROS production of neutrophils. The mean fluorescence intensity (MFI) is shown on the *y*-axis relative to that in unstimulated neutrophils.

Fig. 4

ROS production from HEK293 cells transfected with the mutant Nox2, wt Nox2 or mock plasmid. Open, filled, and hatched bars show ROS production in the absence of PMA, the presence of PMA, and the presence of PMA and DPI, respectively. Note that only HEK293 cells transfected with the wt Nox2 plasmid generated ROS upon stimulation with PMA. Exons 4, 5, and 6 include His101, His115, and His209 + His222 residues, respectively, which are essential for heme-binding. ROS production is expressed as the percentage of that in cells transfected with wt Nox2 in the presence of PMA.

Fig. 5

a Alignment of exon1 to 6 of *CYBB* gene. P1 and P2 are primers for RT-PCR. **b** Schematic representation of splicing pattern of *CYBB* gene transcripts in the CGD patient and healthy control. **c** RT-PCR analysis of total RNA from neutrophils using P1 and P2 primers. The CGD patient under favorable health conditions yielded a 518-bp amplification product that lacked exon 3, whereas the amplification product for healthy control was 629-bp.

Table 1

Laboratory data on admission

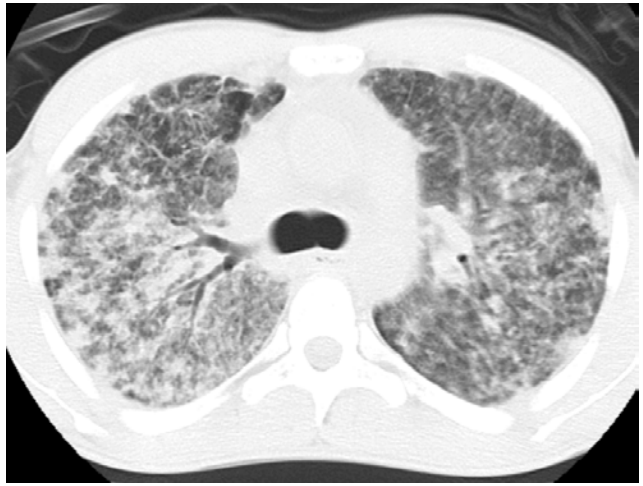
WBC	12,800	/μl
Neutrophils	95	%
Lymphocytes	3	%
Monocytes	1	%
Eosinophils	1	%
RBC	495	$\times 10^4$/μl
Hb	13.4	g/dl
Plt	26	$\times 10^4$/μl
PT	15.5	sec
APTT	30.0	sec
Fib	679	mg/dl
D-dimer	1.5	mg/dl

IgA	551.8	mg/dl
IgG	830.0	mg/dl
IgM	95.3	mg/dl
AST	42	IU/ml
ALT	47	IU/ml
LDH	209	IU/ml
γ-GTP	81	IU/ml
TP	6.0	g/dl
T-Bil	1.0	mg/dl
BUN	14	mg/dl
CRE	0.6	mg/dl
CRP	25.6	mg/dl

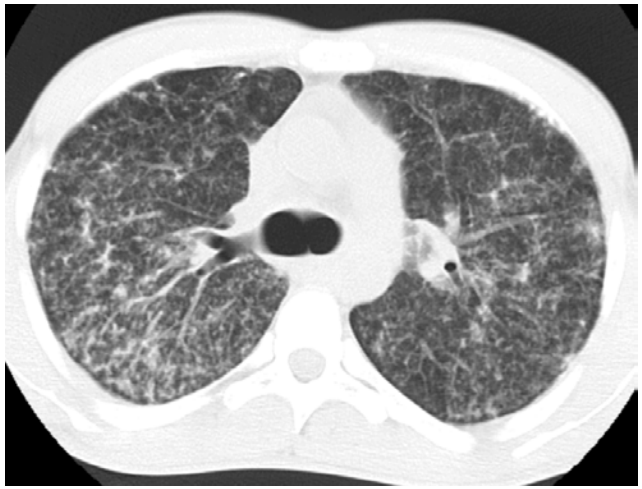
Fig. 1

A

B



C



D

Fig. 2

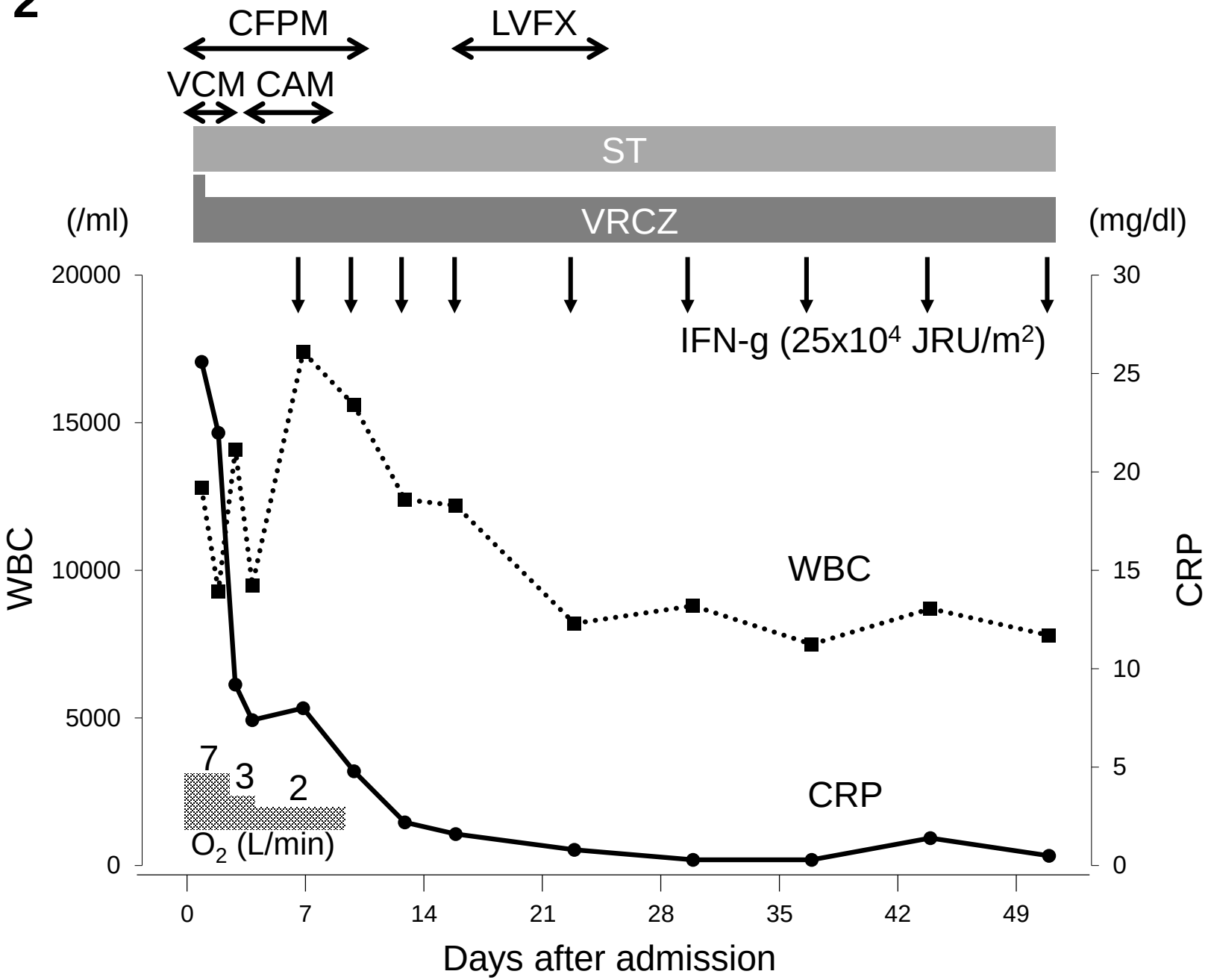


Fig. 3

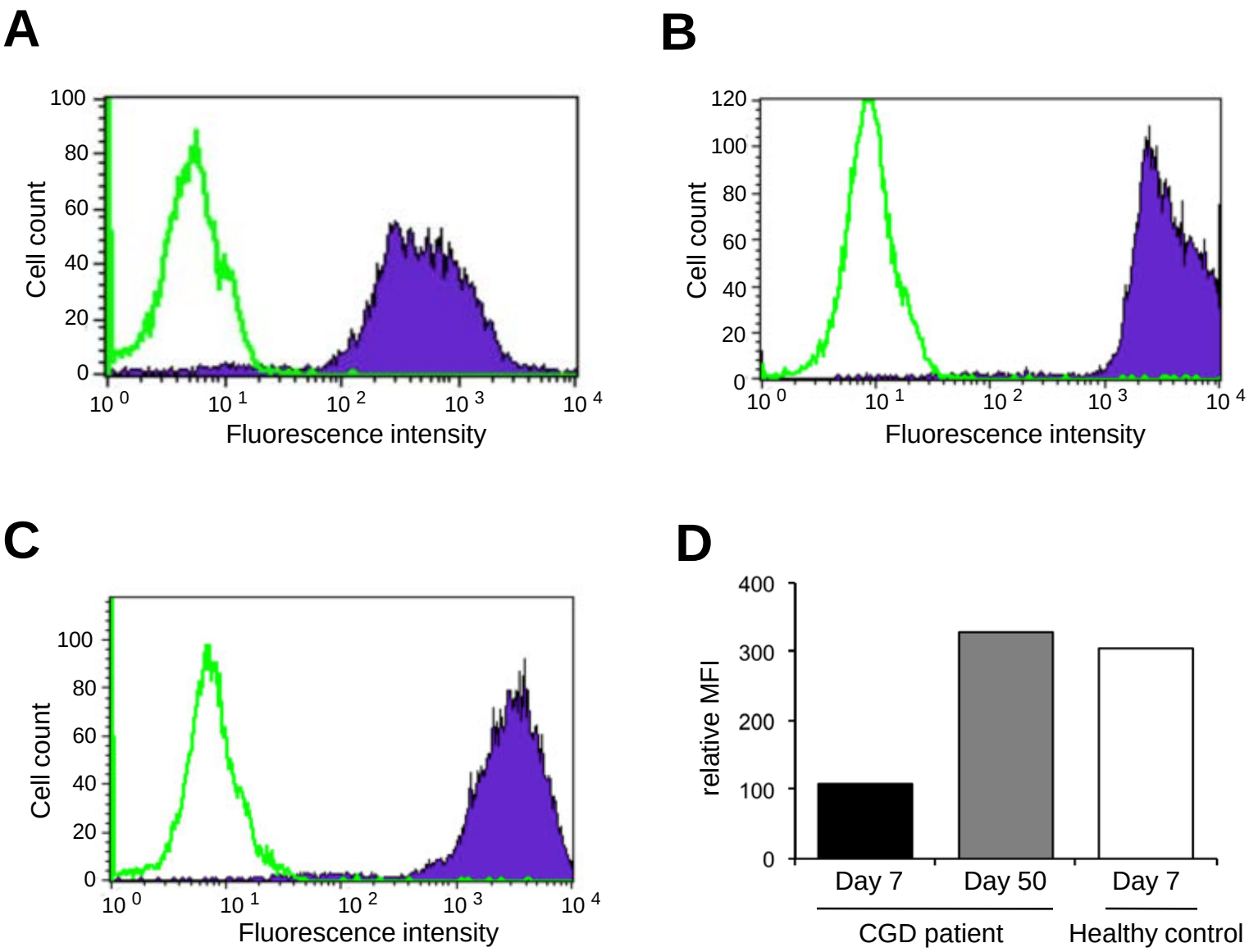


Fig. 4

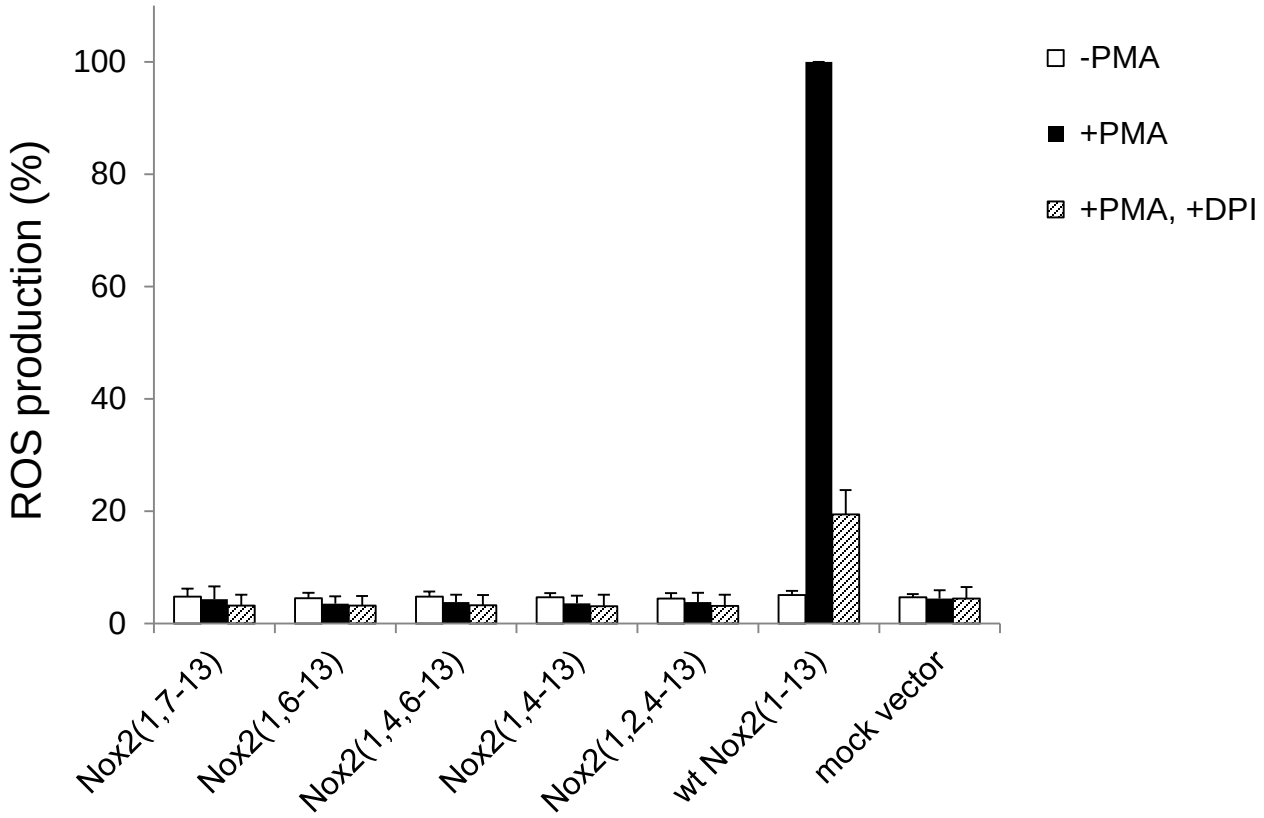


Fig. 5